

APPLICATION  
FOR  
UNITED STATES LETTERS PATENT

TITLE: FATTY ACID DESATURASES AND MUTANT  
SEQUENCES THEREOF

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Express Mail Label No. EV414278223US

January 15, 2004

Date of Deposit

FATTY ACID DESATURASES AND MUTANT SEQUENCES THEREOF

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Cross-Reference to Related Application

This application is a continuation-in-part of PCT Application Serial No. PCT/US96/20090, filed December 13, 1996, which is a continuation-in-part of U.S. Application Serial No. 08/572,027, filed December 14, 1995.

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Technical Field

This invention relates to fatty acid desaturases and nucleic acids encoding desaturase proteins. More particularly, the invention relates to nucleic acids encoding delta-12 and delta-15 fatty acid desaturase proteins that affect fatty acid composition in plants, polypeptides produced from such nucleic acids and plants expressing such nucleic acids.

Background of the Invention

Many breeding studies have been conducted to improve the fatty acid profile of *Brassica* varieties. Pleines and Freidt, *Fat Sci. Technol.*, 90(5), 167-171 (1988) describe plant lines with reduced C<sub>18:3</sub> levels (2.5-5.8%) combined with high oleic content (73-79%). Rakow and McGregor, *J. Amer. Oil Chem. Soc.*, 50, 400-403 (Oct. 1973) discuss problems associated with selecting mutants for linoleic and linolenic acids. In. *Can. J. Plant Sci.*, 68, 509-511 (Apr. 1988) Stellar summer rape producing seed oil with 3% linolenic acid and 28% linoleic acid is disclosed. Roy and Tarr, *Z. Pflanzenzuchtg.*, 95(3), 201-209 (1985) teaches transfer of genes through an interspecific cross from *Brassica juncea* into *Brassica napus* resulting in a reconstituted line combining high linoleic with low linolenic acid content. Roy and Tarr, *Plant Breeding*, 98,

89-96 (1987) discuss prospects for development of *B. napus* L. having improved linolenic and linolenic acid content. European Patent application 323,753 published July 12, 1989 discloses seeds and oils having greater than 79% oleic acid 5 combined with less than 3.5% linolenic acid. Canvin, Can. J. Botany, 43, 63-69 (1965) discusses the effect of temperature on the fatty acid composition of oils from several seed crops including rapeseed.

Mutations typically are induced with extremely high 10 doses of radiation and/or chemical mutagens (Gaul, H. Radiation Botany (1964) 4:155-232). High dose levels which exceed LD50, and typically reach LD90, led to maximum achievable mutation rates. In mutation breeding of *Brassica* varieties high levels of chemical mutagens alone or combined 15 with radiation have induced a limited number of fatty acid mutations (Rakow, G.Z. Pflanzenzuchtg (1973) 69:62-82). The low  $\alpha$ -linolenic acid mutation derived from the Rakow mutation breeding program did not have direct commercial application because of low seed yield. The first commercial 20 cultivar using the low  $\alpha$ -linolenic acid mutation derived in 1973 was released in 1988 as the variety Stellar (Scarth, R. et al., Can. J. Plant Sci. (1988) 68:509-511). Stellar was 20% lower yielding than commercial cultivars at the time of its release.

25 Alterations in fatty acid composition of vegetable oils is desirable for meeting specific food and industrial uses. For example, *Brassica* varieties with increased monounsaturate levels (oleic acid) in the seed oil, and products derived from such oil, would improve lipid 30 nutrition. Canola lines which are low in polyunsaturated fatty acids and high in oleic acid tend to have higher oxidative stability, which is a useful trait for the retail food industry.

Delta-12 fatty acid desaturase (also known as oleic desaturase) is involved in the enzymatic conversion of oleic acid to linoleic acid. Delta-15 fatty acid desaturase (also known as linoleic acid desaturase) is involved in the 5 enzymatic conversion of linoleic acid to  $\alpha$ -linolenic acid. A microsomal delta-12 desaturase has been cloned and characterized using T-DNA tagging. Okuley, et al., Plant Cell 6:147-158 (1994). The nucleotide sequences of higher plant genes encoding microsomal delta-12 fatty acid 10 desaturase are described in Lightner et al., WO94/11516. Sequences of higher plant genes encoding microsomal and plastid delta-15 fatty acid desaturases are disclosed in Yadav, N., et al., Plant Physiol., 103:467-476 (1993), WO 93/11245 and Arondel, V. et al., Science, 258:1353-1355 15 (1992). However, there are no teachings that disclose mutations in delta-12 or delta-15 fatty acid desaturase coding sequences from plants. There is a need in the art for more efficient methods to develop plant lines that contain delta-12 or delta-15 fatty acid desaturase gene 20 sequence mutations effective for altering the fatty acid composition of seeds.

#### Summary of the Invention

The invention comprises *Brassicaceae* or *Helianthus* seeds, plants and plant lines having at least one mutation 25 that controls the levels of unsaturated fatty acids in plants. One embodiment of the invention is an isolated nucleic acid fragment comprising a nucleotide sequence encoding a mutation from a mutant delta-12 fatty acid desaturase conferring altered fatty composition in seeds 30 when the fragment is present in a plant. A preferred sequence comprises a mutant sequence as shown in Fig. 2. Another embodiment of the invention is an isolated nucleic

acid fragment comprising a nucleotide sequence encoding a mutation from a mutant delta-15 fatty acid desaturase. A plant in this embodiment may be soybean, oilseed *Brassica* species, sunflower, castor bean or corn. The mutant 5 sequence may be derived from, for example, a *Brassica napus*, *Brassica rapa*, *Brassica juncea* or *Helianthus* delta-12 or delta-15 desaturase gene.

Another embodiment of the invention involves a method of producing a *Brassicaceae* or *Helianthus* plant line 10 comprising the steps of: (a) inducing mutagenesis in cells of a starting variety of a *Brassicaceae* or *Helianthus* species; (b) obtaining progeny plants from the mutagenized cells; (c) identifying progeny plants that contain a mutation in a delta-12 or delta-15 fatty acid desaturase 15 gene; and (d) producing a plant line by selfing or crossing. The resulting plant line may be subjected to mutagenesis in order to obtain a line having both a delta-12 desaturase mutation and a delta-15 desaturase mutation.

Yet another embodiment of the invention involves a 20 method of producing plant lines containing altered fatty acid composition comprising: (a) crossing a first plant with a second plant having a mutant delta-12 or delta-15 fatty acid desaturase; (b) obtaining seeds from the cross of step (a); (c) growing fertile plants from such seeds; (d) 25 obtaining progeny seed from the plants of step (c); and (e) identifying those seeds among the progeny that have altered fatty acid composition. Suitable plants are soybean, rapeseed, sunflower, safflower, castor bean and corn. Preferred plants are rapeseed and sunflower.

30 The invention is also embodied in vegetable oil obtained from plants disclosed herein, which vegetable oil has an altered fatty acid composition.

Brief Description of the Sequence Listing

SEQ ID NO:1 shows a hypothetical DNA sequence of a *Brassica Fad2* gene. SEQ ID NO:2 is the deduced amino acid sequence of SEQ ID NO:1.

5 SEQ ID NO:3 shows a hypothetical DNA sequence of a *Brassica Fad2* gene having a mutation at nucleotide 316. SEQ ID NO:4 is the deduced amino acid sequence of SEQ ID NO:3.

10 SEQ ID NO:5 shows a hypothetical DNA sequence of a *Brassica Fad2* gene. SEQ ID NO:6 is the deduced amino acid sequence of SEQ ID NO:5.

SEQ ID NO:7 shows a hypothetical DNA sequence of a *Brassica Fad2* gene having a mutation at nucleotide 515. SEQ ID NO:8 is the deduced amino acid sequence of SEQ ID NO:7.

15 SEQ ID NO:9 shows the DNA sequence for the coding region of a wild type *Brassica Fad2-D* gene. SEQ ID NO:10 is the deduced amino acid sequence for SEQ ID NO:9.

SEQ ID NO:11 shows the DNA sequence for the coding region of the IMC 129 mutant *Brassica Fad2-D* gene. SEQ ID NO:12 is the deduced amino acid sequence for SEQ ID NO:11.

20 SEQ ID NO:13 shows the DNA sequence for the coding region of a wild type *Brassica Fad2-F* gene. SEQ ID NO:14 is the deduced amino acid sequence for SEQ ID NO:13.

SEQ ID NO:15 shows the DNA sequence for the coding region of the Q508 mutant *Brassica Fad2-F* gene. SEQ ID NO:16 is the deduced amino acid sequence for SEQ ID NO:15.

25 SEQ ID NO:17 shows the DNA sequence for the coding region of the Q4275 mutant *Brassica Fad2-F* gene. SEQ ID NO:18 is the deduced amino acid sequence for SEQ ID NO:17.

Brief Description of the Figures

30 Figure 1 is a histogram showing the frequency distribution of seed oil oleic acid ( $C_{18:1}$ ) content in a segregating population of a Q508 X Westar cross. The bar

labeled WSGA 1A represents the C<sub>18:1</sub> content of the Westar parent. The bar labeled Q508 represents the C<sub>18:1</sub> content of the Q508 parent.

Figure 2 shows the nucleotide sequences for a 5 *Brassica* Fad2-D wild type gene (Fad2-D wt), IMC129 mutant gene (Fad2-D GA316 IMC129), Fad2-F wild type gene (Fad2-F wt), Q508 mutant gene (Fad2-F TA515 Q508) and Q4275 mutant gene (Fad2-F GA908 Q4275).

Figure 3 shows the deduced amino acid sequences for 10 the polynucleotides of Figure 2.

#### Description of the Preferred Embodiments

All percent fatty acids herein are percent by weight of the oil of which the fatty acid is a component.

As used herein, a "line" is a group of plants that 15 display little or no genetic variation between individuals for at least one trait. Such lines may be created by several generations of self-pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques. As used herein, the term "variety" 20 refers to a line which is used for commercial production.

The term "mutagenesis" refers to the use of a 25 mutagenic agent to induce random genetic mutations within a population of individuals. The treated population, or a subsequent generation of that population, is then screened for usable trait(s) that result from the mutations. A "population" is any group of individuals that share a common gene pool. As used herein "M<sub>0</sub>" is untreated seed. As used herein, "M<sub>1</sub>" is the seed (and resulting plants) exposed to a 30 mutagenic agent, while "M<sub>2</sub>" is the progeny (seeds and plants) of self-pollinated M<sub>1</sub> plants, "M<sub>3</sub>" is the progeny of self-pollinated M<sub>2</sub> plants, and "M<sub>4</sub>" is the progeny of self-pollinated M<sub>3</sub> plants. "M<sub>5</sub>" is the progeny of self-

pollinated  $M_4$  plants. "M<sub>5</sub>", "M<sub>6</sub>", etc. are each the progeny of self-pollinated plants of the previous generation. The term "selfed" as used herein means self-pollinated.

"Stability" or "stable" as used herein means that 5 with respect to a given fatty acid component, the component is maintained from generation to generation for at least two generations and preferably at least three generations at substantially the same level, e.g., preferably  $\pm 5\%$ . The method of invention is capable of creating lines with 10 improved fatty acid compositions stable up to  $\pm 5\%$  from generation to generation. The above stability may be affected by temperature, location, stress and time of planting. Thus, comparison of fatty acid profiles should be made from seeds produced under similar growing conditions. 15 Stability may be measured based on knowledge of prior generation.

Intensive breeding has produced *Brassica* plants whose seed oil contains less than 2% erucic acid. The same varieties have also been bred so that the defatted meal 20 contains less than 30  $\mu\text{mol}$  glucosinolates/gram. "Canola" as used herein refers to plant variety seed or oil which contains less than 2% erucic acid ( $C_{22:1}$ ), and meal with less than 30  $\mu\text{mol}$  glucosinolates/gram.

Applicants have discovered plants with mutations in 25 a delta-12 fatty acid desaturase gene. Such plants have useful alterations in the fatty acid compositions of the seed oil. Such mutations confer, for example, an elevated oleic acid content, a decreased, stabilized linoleic acid content, or both elevated oleic acid and decreased, 30 stabilized linoleic acid content.

Applicants have further discovered plants with mutations in a delta-15 fatty acid desaturase gene. Such plants have useful alterations in the fatty acid composition

of the seed oil, e.g., a decreased, stabilized level of  $\alpha$ -linolenic acid.

Applicants have further discovered isolated nucleic acid fragments (polynucleotides) comprising sequences that 5 carry mutations within the coding sequence of delta-12 or delta-15 fatty acid desaturases. The mutations confer desirable alterations in fatty acid levels in the seed oil of plants carrying such mutations. Delta-12 fatty acid desaturase is also known as omega-6 fatty acid desaturase 10 and is sometimes referred to herein as Fad2 or 12-DES. Delta-15 fatty acid desaturase is also known on omega-3 fatty acid desaturase and is sometimes referred to herein as Fad3 or 15-DES.

A nucleic acid fragment of the invention may be in 15 the form of RNA or in the form of DNA, including cDNA, synthetic DNA or genomic DNA. The DNA may be double-stranded or single-stranded, and if single-stranded, can be either the coding strand or non-coding strand. An RNA analog may be, for example, mRNA or a combination of ribo- 20 and deoxyribonucleotides. Illustrative examples of a nucleic acid fragment of the invention are the mutant sequences shown in Fig. 3.

A nucleic acid fragment of the invention contains a mutation in a microsomal delta-12 fatty acid desaturase 25 coding sequence or a mutation in a microsomal delta-15 fatty acid desaturase coding sequence. Such a mutation renders the resulting desaturase gene product non-functional in plants, relative to the function of the gene product encoded by the wild-type sequence. The non-functionality of the 30 delta-12 desaturase gene product can be inferred from the decreased level of reaction product (linoleic acid) and increased level of substrate (oleic acid) in plant tissues expressing the mutant sequence, compared to the

corresponding levels in plant tissues expressing the wild-type sequence. The non-functionality of the delta-15 desaturase gene product can be inferred from the decreased level of reaction product ( $\alpha$ -linolenic acid) and the 5 increased level of substrate (linoleic acid) in plant tissues expressing the mutant sequence, compared to the corresponding levels in plant tissues expressing the wild-type sequence.

A nucleic acid fragment of the invention may 10 comprise a portion of the coding sequence, e.g., at least about 10 nucleotides, provided that the fragment contains at least one mutation in the coding sequence. The length of a desired fragment depends upon the purpose for which the fragment will be used, e.g., PCR primer, site-directed 15 mutagenesis and the like. In one embodiment, a nucleic acid fragment of the invention comprises the full length coding sequence of a mutant delta-12 or mutant delta-15 fatty acid desaturase, e.g., the mutant sequences of Fig. 3. In other embodiments, a nucleic acid fragment is about 20 to about 50 20 nucleotides (or base pairs, bp), or about 50 to about 500 nucleotides, or about 500 to about 1200 nucleotides in length.

A mutation in a nucleic acid fragment of the invention may be in any portion of the coding sequence that 25 renders the resulting gene product non-functional. Suitable types of mutations include, without limitation, insertions of nucleotides, deletions of nucleotides, or transitions and transversions in the wild-type coding sequence. Such mutations result in insertions of one or more amino acids, 30 deletions of one or more amino acids, and non-conservative amino acid substitutions in the corresponding gene product. In some embodiments, the sequence of a nucleic acid fragment

may comprise more than one mutation or more than one type of mutation.

Insertion or deletion of amino acids in a coding sequence may, for example, disrupt the conformation of 5 essential alpha-helical or beta-pleated sheet regions of the resulting gene product. Amino acid insertions or deletions may also disrupt binding or catalytic sites important for gene product activity. It is known in the art that the insertion or deletion of a larger number of contiguous amino 10 acids is more likely to render the gene product non-functional, compared to a smaller number of inserted or deleted amino acids.

Non-conservative amino acid substitutions may 15 replace an amino acid of one class with an amino acid of a different class. Non-conservative substitutions may make a substantial change in the charge or hydrophobicity of the gene product. Non-conservative amino acid substitutions may also make a substantial change in the bulk of the residue side chain, e.g., substituting an alanyl residue for a 20 isoleucyl residue.

Examples of non-conservative substitutions include the substitution of a basic amino acid for a non-polar amino acid, or a polar amino acid for an acidic amino acid. Because there are only 20 amino acids encoded in a gene, 25 substitutions that result in a non-functional gene product may be determined by routine experimentation, incorporating amino acids of a different class in the region of the gene product targeted for mutation.

Preferred mutations are in a region of the nucleic acid 30 encoding an amino acid sequence motif that is conserved among delta-12 fatty acid desaturases or delta-15 fatty acid desaturases, such as a His-Xaa-Xaa-Xaa-His motif (Tables 1-3). An example of a suitable region has a conserved HECGH

motif that is found, for example, in nucleotides corresponding to amino acids 105 to 109 of the *Arabidopsis* and *Brassica* delta-12 desaturase sequences, in nucleotides corresponding to amino acids 101 to 105 of the soybean 5 delta-12 desaturase sequence and in nucleotides corresponding to amino acids 111 to 115 of the maize delta-12 desaturase sequence. See e.g., WO 94/115116; Okuley et al., *Plant Cell* 6:147-158 (1994). The one letter amino acid designations used herein are described in Alberts, B. et 10 al., *Molecular Biology of the Cell*, 3rd edition, Garland Publishing, New York, 1994. Amino acids flanking this motif are also highly conserved among delta-12 and delta-15 desaturases and are also suitable candidates for mutations in fragments of the invention.

15 An illustrative embodiment of a mutation in a nucleic acid fragment of the invention is a Glu to Lys substitution in the HECGH motif of a *Brassica* microsomal delta-12 desaturase sequence, either the D form or the F form. This mutation results in the sequence HECGH being 20 changed to HKCGH as seen by comparing SEQ ID NO:10 (wild-type D form) to SEQ ID NO:12 (mutant D form). A similar mutation in other Fad-2 sequences is contemplated to result in a non-functional gene product. (Compare SEQ ID NO:2 to SEQ ID NO:4).

25 A similar motif may be found at amino acids 101 to 105 of the *Arabidopsis* microsomal delta-15 fatty acid desaturase, as well as in the corresponding rape and soybean desaturases (Table 5). See, e.g., WO 93/11245; Arondel, V. et al., *Science*, 258:1153-1155 (1992); Yadav, N. et al., 30 *Plant Physiol.*, 103:467-476 (1993). Plastid delta-15 fatty acids have a similar motif (Table 5).

Among the types of mutations in an HECGH motif that render the resulting gene product non-functional are non-

conservative substitutions. An illustrative example of a non-conservative substitution is substitution of a glycine residue for either the first or second histidine. Such a substitution replaces a charged residue (histidine) with a 5 non-polar residue (glycine). Another type of mutation that renders the resulting gene product non-functional is an insertion mutation, e.g., insertion of a glycine between the cysteine and glutamic acid residues in the HECGH motif.

Other regions having suitable conserved amino acid 10 motifs include the HRRHH motif shown in Table 2, the HRTHH motif shown in Table 6 and the HVAHH motif shown in Table 3. See, e.g., WO 94/115116; Hitz, W. et al., *Plant Physiol.*, 105:635-641 (1994); Okuley, J., et al., *supra*; and Yadav, N. et al., *supra*. An illustrative example of a mutation in the 15 region shown in Table 3 is a mutation at nucleotides corresponding to the codon for glycine (amino acid 303 of *B. napus*). A non-conservative Gly to Glu substitution results in the amino acid sequence DRDYGILNKV being changed to sequence DRDYEILNKV (compare wild-type F form SEQ ID NO:14 20 to mutant Q4275 SEQ ID NO:18, Fig. 3).

Another region suitable for a mutation in a delta-12 desaturase sequence contains the motif KYLNNP at nucleotides corresponding to amino acids 171 to 175 of the *Brassica* desaturase sequence. An illustrative example of a mutation 25 in this region is a Leu to His substitution, resulting in the amino acid sequence (Table 4) KYHNN (compare wild-type Fad2-F SEQ ID NO:14 to mutant SEQ ID NO:16). A similar mutation in other Fad-2 amino acid sequences is contemplated to result in a non-functional gene product. (Compare SEQ ID 30 NO:6 to SEQ ID NO:8).

TABLE 1

Alignment of Amino Acid Sequences from Microsomal  
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
5	<i>Arabidopsis thaliana</i>	100-129	IWVIAHECGH HAFSDYQWLD DTVGLIFHSF
	<i>Glycine max</i>	96-125	VWVIAHECGH HAFSKYQWVD DVVGLTLHST
	<i>Zea mays</i>	106-135	VWVIAHECGH HAFSDYSLLD DVVGLVLHSS
	<i>Ricinus communis</i> <sup>a</sup>	1- 29	WVMAHDCGH HAFSDYQLLD DVVGLILHSC
10	<i>Brassica napus D</i>	100-128	VWVIAHECGH HAFSDYQWLD DTVGLIFHS
	<i>Brassica napus F</i>	100-128	VWVIAHECGH HAFSDYQWLD DTVGLIFHS

<sup>a</sup> from plasmid pRF2-1C

TABLE 2

Alignment of Amino Acid Sequences from Microsomal  
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
15	<i>Arabidopsis thaliana</i>	130-158	LLVPYFSWKY SHRRHHSNTG SLERDEVFV
	<i>Glycine max</i>	126-154	LLVPYFSWKI SHRRHHSNTG SLDRDEVFV
	<i>Zea mays</i>	136-164	LMVPYFSWKY SHRRHHSNTG SLERDEVFV
	<i>Ricinus communis</i> <sup>a</sup>	30- 58	LLVPYFSWKH SHRRHHSNTG SLERDEVFV
20	<i>Brassica napus D</i>	130-158	LLVPYFSWKY SHRRHHSNTG SLERDEVFV
	<i>Brassica napus F</i>	130-158	LLVPYFSWKY SHRRHHSNTG SLERDEVFV

<sup>a</sup> from plasmid pRF2-1C

TABLE 3

Alignment of Amino Acid Sequences from Microsomal  
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
25	<i>Arabidopsis thaliana</i>	298-333	DRDYGILNKV FHNITDTHVA HHLFSTMPHY NAMEAT
	<i>Glycine max</i>	294-329	DRDYGILNKV FHHITDTHVA HHLFSTMPHY HAMEAT
	<i>Zea mays</i>	305-340	DRDYGILNRV FHNITDTHVA HHLFSTMPHY HAMEAT
30	<i>Ricinus communis</i> <sup>a</sup>	198-224	DRDYGILNKV FHNITDTQVA HHLF TMP
	<i>Brassica napus D</i>	299-334	DRDYGILNKV FHNITDTHVA HHLFSTMPHY HAMEAT
	<i>Brassica napus F</i>	299-334	DRDYGILNKV FHNITDTHVA HHLFSTMPHY HAMEAT

<sup>a</sup> from plasmid pRF2-1C

TABLE 4

Alignment of Conserved Amino Acids from Microsomal  
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
5	<i>Arabidopsis thaliana</i>	165-180	IKWYGKYLNN PLGRIM
	<i>Glycine max</i>	161-176	VAWFSLYLN PLGRAV
	<i>Zea mays</i>	172-187	PWYTPYVYNN PVGRVV
	<i>Ricinus communis</i> <sup>a</sup>	65- 80	IRWYSKYLNN PPGRIM
	<i>Brassica napus</i> D	165-180	IKWYGKYLNN PLGRTV
10	<i>Brassica napus</i> F	165-180	IKWYGKYLNN PLGRTV

<sup>a</sup> from plasmid pRF2-1C

TABLE 5

Alignment of Conserved Amino Acids from Plastid and Microsomal  
Delta-15 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
15	<i>Arabidopsis thaliana</i> <sup>a</sup>	156-177	WALFVLGHD CGHGSFSNDP KLN
	<i>Brassica napus</i> <sup>a</sup>	114-135	WALFVLGHD CGHGSFSNDP RLN
	<i>Glycine max</i> <sup>a</sup>	164-185	WALFVLGHD CGHGSFSNNNS KLN
	<i>Arabidopsis thaliana</i>	94-115	WAIFVLGHD CGHGSFSDIP LLN
20	<i>Brassica napus</i>	87-109	WALFVLGHD CGHGSFSNDP RLN
	<i>Glycine max</i>	93-114	WALFVLGHD CGHGSFSDSP PLN

<sup>a</sup> Plastid sequences

TABLE 6

Alignment of Conserved Amino Acids from Plastid and Microsomal  
Delta-15 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
25	<i>A. thaliana</i> <sup>a</sup>	188-216	ILVPYHGWRI SHRTHHQNHG HVENDESWH
	<i>B. napus</i> <sup>a</sup>	146-174	ILVPYHGWRI SHRTHHQNHG HVENDESWH
	<i>Glycine max</i> <sup>a</sup>	196-224	ILVPYHGWRI SHRTHHQNHG HAENDESWH
30	<i>A. thaliana</i>	126-154	ILVPYHGWRI SHRTHHQNHG HVENDESWV
	<i>Brassica napus</i>	117-145	ILVPYHGWRI SHRTHHQNHG HVENDESWV
	<i>Glycine max</i>	125-153	ILVPYHGWRI SHRTHHQNHG HIEKDESWV

<sup>a</sup> Plastid sequences

The conservation of amino acid motifs and their  
35 relative positions indicates that regions of a delta-12 or

delta-15 fatty acid desaturase that can be mutated in one species to generate a non-functional desaturase can be mutated in the corresponding region from other species to generate a non-functional delta-12 desaturase or delta-15 desaturase gene product in that species.

Mutations in any of the regions of Tables 1-6 are specifically included within the scope of the invention and are substantially identical to those mutations exemplified herein, provided that such mutation (or mutations) renders the resulting desaturase gene product non-functional, as discussed hereinabove.

A nucleic acid fragment containing a mutant sequence can be generated by techniques known to the skilled artisan. Such techniques include, without limitation, site-directed mutagenesis of wild-type sequences and direct synthesis 15 using automated DNA synthesizers.

A nucleic acid fragment containing a mutant sequence can also be generated by mutagenesis of plant seeds or regenerable plant tissue by, e.g., ethyl methane sulfonate, 20 X-rays or other mutagens. With mutagenesis, mutant plants having the desired fatty acid phenotype in seeds are identified by known techniques and a nucleic acid fragment containing the desired mutation is isolated from genomic DNA or RNA of the mutant line. The site of the specific 25 mutation is then determined by sequencing the coding region of the delta-12 desaturase or delta-15 desaturase gene. Alternatively, labeled nucleic acid probes that are specific for desired mutational events can be used to rapidly screen a mutagenized population.

30 The disclosed method may be applied to all oilseed *Brassica* species, and to both Spring and Winter maturing types within each species. Physical mutagens, including but not limited to X-rays, UV rays, and other physical

treatments which cause chromosome damage, and other chemical mutagens, including but not limited to ethidium bromide, nitrosoguanidine, diepoxybutane etc. may also be used to induce mutations. The mutagenesis treatment may also be 5 applied to other stages of plant development, including but not limited to cell cultures, embryos, microspores and shoot apices.

"Stable mutations" as used herein are defined as  $M_5$  or more advanced lines which maintain a selected altered 10 fatty acid profile for a minimum of three generations, including a minimum of two generations under field conditions, and exceeding established statistical thresholds for a minimum of two generations, as determined by gas chromatographic analysis of a minimum of 10 randomly 15 selected seeds bulked together. Alternatively, stability may be measured in the same way by comparing to subsequent generations. In subsequent generations, stability is defined as having similar fatty acid profiles in the seed as that of the prior or subsequent generation when grown under 20 substantially similar conditions.

Mutation breeding has traditionally produced plants carrying, in addition to the trait of interest, multiple, deleterious traits, e.g., reduced plant vigor and reduced fertility. Such traits may indirectly affect fatty acid 25 composition, producing an unstable mutation; and/or reduce yield, thereby reducing the commercial utility of the invention. To eliminate the occurrence of deleterious mutations and reduce the load of mutations carried by the plant, a low mutagen dose is used in the seed treatments to 30 create an LD30 population. This allows for the rapid selection of single gene mutations for fatty acid traits in agronomic backgrounds which produce acceptable yields.

The seeds of several different fatty acid lines have been deposited with the American Type Culture Collection and have the following accession numbers.

	<u>Line</u>	<u>Accession No.</u>	<u>Deposit Date</u>
5	A129.5	40811	May 25, 1990
	A133.1	40812	May 25, 1990
	M3032.1	75021	June 7, 1991
	M3062.8	75025	June 7, 1991
	M3028.10	75026	June 7, 1991
10	IMC130	75446	April 16, 1993
	Q4275	97569	May 10, 1996

In some plant species or varieties more than one form of endogenous microsomal delta-12 desaturase may be found. In amphidiploids, each form may be derived from one 15 of the parent genomes making up the species under consideration. Plants with mutations in both forms have a fatty acid profile that differs from plants with a mutation in only one form. An example of such a plant is *Brassica napus* line Q508, a doubly-mutagenized line containing a 20 mutant D-form of delta-12 desaturase (SEQ ID NO:11) and a mutant F-form of delta-12 desaturase (SEQ ID NO:15). Another example is line Q4275, which contains a mutant D-form of delta-12 desaturase (SEQ ID NO:11) and a mutant F-form of delta-12 desaturase (SEQ ID NO:17). See Figs. 2-3.

25 Preferred host or recipient organisms for introduction of a nucleic acid fragment of the invention are the oil-producing species, such as soybean (*Glycine max*), rapeseed (e.g., *Brassica napus*, *B. rapa* and *B. juncea*), sunflower (*Helianthus annus*), castor bean (*Ricinus communis*), corn (*Zea mays*), and safflower (*Carthamus tinctorius*).

A nucleic acid fragment of the invention may further comprise additional nucleic acids. For example, a nucleic acid encoding a secretory or leader amino acid sequence can

be linked to a mutant desaturase nucleic acid fragment such that the secretory or leader sequence is fused in-frame to the amino terminal end of a mutant delta-12 or delta-15 desaturase polypeptide. Other nucleic acid fragments are 5 known in the art that encode amino acid sequences useful for fusing in-frame to the mutant desaturase polypeptides disclosed herein. See, e.g., U.S. 5,629,193 incorporated herein by reference. A nucleic acid fragment may also have one or more regulatory elements operably linked thereto.

10 The present invention also comprises nucleic acid fragments that selectively hybridize to mutant desaturase sequences. Such a nucleic acid fragment typically is at least 15 nucleotides in length. Hybridization typically involves Southern analysis (Southern blotting), a method by 15 which the presence of DNA sequences in a target nucleic acid mixture are identified by hybridization to a labeled oligonucleotide or DNA fragment probe. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after 20 electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook et al., (1989) *Molecular Cloning*, second edition, Cold 25 Spring Harbor Laboratory, Plainview, NY.

A nucleic acid fragment can hybridize under moderate stringency conditions or, preferably, under high stringency conditions to a mutant desaturase sequence. High stringency conditions are used to identify nucleic acids that have a 30 high degree of homology to the probe. High stringency conditions can include the use of low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate (0.1X SSC); 0.1% sodium lauryl

sulfate (SDS) at 50-65°C. Alternatively, a denaturing agent such as formamide can be employed during hybridization, e.g., 50% formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is the use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 10 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

Moderate stringency conditions refers to hybridization conditions used to identify nucleic acids that have a lower degree of identity to the probe than do nucleic acids identified under high stringency conditions. Moderate stringency conditions can include the use of higher ionic strength and/or lower temperatures for washing of the hybridization membrane, compared to the ionic strength and temperatures used for high stringency hybridization. For example, a wash solution comprising 0.060 M NaCl/0.0060 M sodium citrate (4X SSC) and 0.1% sodium lauryl sulfate (SDS) can be used at 50°C, with a last wash in 1X SSC, at 65°C. Alternatively, a hybridization wash in 1X SSC at 37°C can be used.

25 Hybridization can also be done by Northern analysis (Northern blotting), a method used to identify RNAs that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as  $^{32}\text{P}$ , by 30 biotinylation or with an enzyme. The RNA to be analyzed can be usually electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe,

using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook et al., *supra*.

A polypeptide of the invention comprises an isolated 5 polypeptide having a mutant amino acid sequence, as well as derivatives and analogs thereof. See, e.g., the mutant amino acid sequences of Fig. 3. By "isolated" is meant a polypeptide that is expressed and produced in an environment other than the environment in which the polypeptide is 10 naturally expressed and produced. For example, a plant polypeptide is isolated when expressed and produced in bacteria or fungi. A polypeptide of the invention also comprises variants of the mutant desaturase polypeptides disclosed herein, as discussed above.

15 In one embodiment of the claimed invention, a plant contains both a delta-12 desaturase mutation and a delta-15 desaturase mutation. Such plants can have a fatty acid composition comprising very high oleic acid and very low alpha-linolenic acid levels. Mutations in delta-12 20 desaturase and delta-15 desaturase may be combined in a plant by making a genetic cross between delta-12 desaturase and delta-15 desaturase single mutant lines. A plant having a mutation in delta-12 fatty acid desaturase is crossed or mated with a second plant having a mutation in delta-15 25 fatty acid desaturase. Seeds produced from the cross are planted and the resulting plants are selfed in order to obtain progeny seeds. These progeny seeds are then screened in order to identify those seeds carrying both mutant genes.

30 Alternatively, a line possessing either a delta-12 desaturase or a delta-15 desaturase mutation can be subjected to mutagenesis to generate a plant or plant line having mutations in both delta-12 desaturase and delta-15 desaturase. For example, the IMC 129 line has a mutation in

the coding region (Glu<sub>106</sub> to Lys<sub>106</sub>) of the D form of the microsomal delta-12 desaturase structural gene. Cells (e.g., seeds) of this line can be mutagenized to induce a mutation in a delta-15 desaturase gene, resulting in a plant 5 or plant line carrying a mutation in a delta-12 fatty acid desaturase gene and a mutation in a delta-15 fatty acid desaturase gene.

Progeny includes descendants of a particular plant or plant line, e.g., seeds developed on an instant plant are 10 descendants. Progeny of an instant plant include seeds formed on F<sub>1</sub>, F<sub>2</sub>, F<sub>3</sub>, and subsequent generation plants, or seeds formed on BC<sub>1</sub>, BC<sub>2</sub>, BC<sub>3</sub>, and subsequent generation plants.

Plants according to the invention preferably contain 15 an altered fatty acid composition. For example, oil obtained from seeds of such plants may have from about 69 to about 90% oleic acid, based on the total fatty acid composition of the seed. Such oil preferably has from about 74 to about 90% oleic acid, more preferably from about 80 to 20 about 90% oleic acid. In some embodiments, oil obtained from seeds produced by plants of the invention may have from about 2.0% to about 5.0% saturated fatty acids, based on total fatty acid composition of the seeds. In some 25 embodiments, oil obtained from seeds of the invention may have from about 1.0% to about 14.0% linoleic acid, or from about 0.5% to about 10.0%  $\alpha$ -linolenic acid.

Oil composition typically is analyzed by crushing and extracting fatty acids from bulk seed samples (e.g., 10 seeds). Fatty acid triglycerides in the seed are hydrolyzed 30 and converted to fatty acid methyl esters. Those seeds having an altered fatty acid composition may be identified by techniques known to the skilled artisan, e.g., gas-liquid chromatography (GLC) analysis of a bulked seed sample or of

a single half-seed. Half-seed analysis is well known in the art to be useful because the viability of the embryo is maintained and thus those seeds having a desired fatty acid profile may be planted to from the next generation.

5 However, half-seed analysis is also known to be an inaccurate representation of genotype of the seed being analyzed. Bulk seed analysis typically yields a more accurate representation of the fatty acid profile of a given genotype. Fatty acid composition can also be determined on  
10 larger samples, e.g., oil obtained by pilot plant or commercial scale refining, bleaching and deodorizing of endogenous oil in the seeds.

The nucleic acid fragments of the invention can be used as markers in plant genetic mapping and plant breeding  
15 programs. Such markers may include restriction fragment length polymorphism (RFLP), random amplification polymorphism detection (RAPD), polymerase chain reaction (PCR) or self-sustained sequence replication (3SR) markers, for example. Marker-assisted breeding techniques may be  
20 used to identify and follow a desired fatty acid composition during the breeding process. Marker-assisted breeding techniques may be used in addition to, or as an alternative to, other sorts of identification techniques. An example of marker-assisted breeding is the use of PCR primers that  
25 specifically amplify a sequence containing a desired mutation in delta-12 desaturase or delta-15 desaturase.

Methods according to the invention are useful in that the resulting plants and plant lines have desirable seed fatty acid compositions as well as superior agronomic properties compared to known lines having altered seed fatty acid composition. Superior agronomic characteristics include, for example, increased seed germination percentage, increased seedling vigor, increased resistance to seedling

fungal diseases (damping off, root rot and the like), increased yield, and improved standability.

While the invention is susceptible to various modifications and alternative forms, certain specific 5 embodiments thereof are described in the general methods and examples set forth below. For example the invention may be applied to all *Brassica* species, including *B. rapa*, *B. juncea*, and *B. hirta*, to produce substantially similar results. It should be understood, however, that these 10 examples are not intended to limit the invention to the particular forms disclosed but, instead the invention is to cover all modifications, equivalents and alternatives falling within the scope of the invention. This includes the use of somaclonal variation; physical or chemical 15 mutagenesis of plant parts; anther, microspore or ovary culture followed by chromosome doubling; or self- or cross-pollination to transmit the fatty acid trait, alone or in combination with other traits, to develop new *Brassica* lines.

20

#### EXAMPLE 1

##### Mutagenesis

Seeds of Westar, a Canadian (*Brassica napus*) spring canola variety, were subjected to chemical mutagenesis.

Westar is a registered Canadian spring variety with canola 25 quality. The fatty acid composition of field-grown Westar, 3.9% C<sub>16:0</sub>, 1.9% C<sub>18:0</sub>, 67.5% C<sub>18:1</sub>, 17.6% C<sub>18:2</sub>, 7.4% C<sub>18:3</sub>, <2% C<sub>20:1</sub> + C<sub>22:1</sub>, has remained stable under commercial production, with  $\pm$  10% deviation, since 1982.

Prior to mutagenesis, 30,000 seeds of *B. napus* cv. 30 Westar seeds were preimbibed in 300-seed lots for two hours on wet filter paper to soften the seed coat. The preimbibed seeds were placed in 80 mM ethylmethanesulfonate (EMS) for

four hours. Following mutagenesis, the seeds were rinsed three times in distilled water. The seeds were sown in 48-well flats containing Pro-Mix. Sixty-eight percent of the mutagenized seed germinated. The plants were maintained at 5 25°C/15°C, 14/10 hr day/night conditions in the greenhouse. At flowering, each plant was individually self-pollinated.

M<sub>2</sub> seed from individual plants were individually catalogued and stored, approximately 15,000 M<sub>2</sub> lines was planted in a summer nursery in Carman, Manitoba. The seed 10 from each selfed plant were planted in 3-meter rows with 6-inch row spacing. Westar was planted as the check variety. Selected lines in the field were selfed by bagging the main raceme of each plant. At maturity, the selfed plants were individually harvested and seeds were catalogued and stored 15 to ensure that the source of the seed was known.

Self-pollinated M<sub>3</sub> seed and Westar controls were analyzed in 10-seed bulk samples for fatty acid composition via gas chromatography. Statistical thresholds for each fatty acid component were established using a Z-distribution 20 with a stringency level of 1 in 10,000. Mean and standard deviation values were determined from the non-mutagenized Westar control population in the field. The upper and lower statistical thresholds for each fatty acid were determined from the mean value of the population  $\pm$  the standard deviation, multiplied by the Z-distribution. Based on a 25 population size of 10,000, the confidence interval is 99.99%.

The selected M<sub>3</sub> seeds were planted in the greenhouse along with Westar controls. The seed was sown in 4-inch 30 pots containing Pro-Mix soil and the plants were maintained at 25°C/15°C, 14/10 hr day/night cycle in the greenhouse. At flowering, the terminal raceme was self-pollinated by bagging. At maturity, selfed M<sub>4</sub> seed was individually

harvested from each plant, labelled, and stored to ensure that the source of the seed was known.

The  $M_4$  seed was analyzed in 10-seed bulk samples. Statistical thresholds for each fatty acid component were 5 established from 259 control samples using a Z-distribution of 1 in 800. Selected  $M_4$  lines were planted in a field trial in Carman, Manitoba in 3-meter rows with 6-inch spacing. Ten  $M_4$  plants in each row were bagged for self-pollination. At maturity, the selfed plants were 10 individually harvested and the open pollinated plants in the row were bulk harvested. The  $M_5$  seed from single plant selections was analyzed in 10-seed bulk samples and the bulk row harvest in 50-seed bulk samples.

Selected  $M_5$  lines were planted in the greenhouse 15 along with Westar controls. The seed was grown as previously described. At flowering the terminal raceme was self-pollinated by bagging. At maturity, selfed  $M_6$  seed was individually harvested from each plant and analyzed in 10-seed bulk samples for fatty acid composition.

Selected  $M_6$  lines were entered into field trials in 20 Eastern Idaho. The four trial locations were selected for the wide variability in growing conditions. The locations included Burley, Teton, Lamont and Shelley (Table 7). The lines were planted in four 3-meter rows with an 8-inch spacing, each plot was replicated four times. The planting 25 design was determined using a Randomized Complete Block Designed. The commercial cultivar Westar was used as a check cultivar. At maturity the plots were harvested to determine yield. Yield of the entries in the trial was 30 determined by taking the statistical average of the four replications. The Least Significant Difference Test was used to rank the entries in the randomized complete block design.

TABLE 7  
Trial Locations for Selected Fatty Acid Mutants

<u>LOCATION</u>		<u>SITE CHARACTERIZATIONS</u>
BURLEY		Irrigated. Long season. High temperatures during flowering.
5 TETONIA		Dryland. Short season. Cool temperatures.
LAMONT		Dryland. Short season. Cool temperatures.
SHELLEY		Irrigated. Medium season. High temperatures during flowering.

10 To determine the fatty acid profile of entries, plants in each plot were bagged for self-pollination. The  $M_7$  seed from single plants was analyzed for fatty acids in ten-seed bulk samples.

15 To determine the genetic relationships of the selected fatty acid mutants crosses were made. Flowers of  $M_6$  or later generation mutations were used in crossing.  $F_1$  seed was harvested and analyzed for fatty acid composition to determine the mode of gene action. The  $F_1$  progeny were planted in the greenhouse. The resulting plants were self-pollinated, the  $F_2$  seed harvested and analyzed for fatty acid composition for allelism studies. The  $F_2$  seed and 20 parent line seed was planted in the greenhouse, individual plants were self-pollinated. The  $F_3$  seed of individual plants was tested for fatty acid composition using 10-seed bulk samples as described previously.

25 In the analysis of some genetic relationships dihaploid populations were made from the microspores of the  $F_1$  hybrids. Self-pollinated seed from dihaploid plants were analyzed for fatty acid analysis using methods described previously.

30 For chemical analysis, 10-seed bulk samples were hand ground with a glass rod in a 15-mL polypropylene tube

and extracted in 1.2 mL 0.25 N KOH in 1:1 ether/methanol. The sample was vortexed for 30 sec. and heated for 60 sec. in a 60°C water bath. Four mL of saturated NaCl and 2.4 mL of iso-octane were added, and the mixture was vortexed 5 again. After phase separation, 600  $\mu$ L of the upper organic phase were pipetted into individual vials and stored under nitrogen at -5°C. One  $\mu$ L samples were injected into a Supelco SP-2330 fused silica capillary column (0.25 mm ID, 30 M length, 0.20  $\mu$ m df).

10 The gas chromatograph was set at 180°C for 5.5 minutes, then programmed for a 2°C/minute increase to 212°C, and held at this temperature for 1.5 minutes. Total run time was 23 minutes. Chromatography settings were: Column head pressure - 15 psi, Column flow (He) - 0.7 mL/min., 15 Auxiliary and Column flow - 33 mL/min., Hydrogen flow - 33 mL/min., Air flow - 400 mL/min., Injector temperature - 250°C, Detector temperature - 300°C, Split vent - 1/15.

Table 8 describes the upper and lower statistical thresholds for each fatty acid of interest.

**TABLE 8**  
 Statistical Thresholds for Specific Fatty Acids  
Derived from Control Westar Plantings

5	Genotype	Percent Fatty Acids					
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	Sats*
<b>M<sub>3</sub> Generation (1 in 10,000 rejection rate)</b>							
	Lower	3.3	1.4	--	13.2	5.3	6.0
	Upper	4.3	2.5	71.0	21.6	9.9	8.3
<b>M<sub>4</sub> Generation (1 in 800 rejection rate)</b>							
10	Lower	3.6	0.8	--	12.2	3.2	5.3
	Upper	6.3	3.1	76.0	32.4	9.9	11.2
<b>M<sub>5</sub> Generation (1 in 755 rejection rate)</b>							
	Lower	2.7	0.9	--	9.6	2.6	4.5
15	Upper	5.7	2.7	80.3	26.7	9.6	10.0

\*Sats=Total Saturate Content

EXAMPLE 2

High Oleic Acid Canola Lines

In the studies of Example 1, at the M<sub>3</sub> generation,  
 20 31 lines exceeded the upper statistical threshold for oleic acid ( $\geq 71.0\%$ ). Line W7608.3 had 71.2% oleic acid. At the M<sub>4</sub> generation, its selfed progeny (W7608.3.5, since designated A129.5) continued to exceed the upper statistical threshold for C<sub>18:1</sub> with 78.8% oleic acid. M<sub>5</sub> seed of five  
 25 self-pollinated plants of line A129.5 (ATCC 40811) averaged 75.0% oleic acid. A single plant selection, A129.5.3 had 75.6% oleic acid. The fatty acid composition of this high oleic acid mutant, which was stable under both field and greenhouse conditions to the M<sub>5</sub> generation, is summarized in

Table 9. This line also stably maintained its mutant fatty acid composition to the M<sub>5</sub> generation in field trials in multiple locations. Over all locations the self-pollinated plants (A129) averaged 78.3% oleic acid. The fatty acid composition of the A129 for each Idaho trial location are summarized in Table 10. In multiple location replicated yield trials, A129 was not significantly different in yield from the parent cultivar Westar.

The canola oil of A129, after commercial processing, was found to have superior oxidative stability compared to Westar when measured by the Accelerated Oxygen Method (AOM), American Oil Chemists' Society Official Method Cd 12-57 for fat stability; Active Oxygen Method (revised 1989). The AOM of Westar was 18 AOM hours and for A129 was 30 AOM hours.

15

**TABLE 9**

**Fatty Acid Composition of a High Oleic Acid Canola Line Produced by Seed Mutagenesis**

	<b>Genotype</b>	<b>Percent Fatty Acids</b>					
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	Sats
20	Westar	3.9	1.9	67.5	17.6	7.4	7.0
	W7608.3 (M <sub>3</sub> )	3.9	2.4	71.2	12.7	6.1	7.6
	W7608.3.5 (M <sub>4</sub> )	3.9	2.0	78.8	7.7	3.9	7.3
25	A129.5.3 (M <sub>5</sub> )	3.8	2.3	75.6	9.5	4.9	7.6

Sats=Total Saturate Content

TABLE 10

Fatty Acid Composition of a Mutant High  
Oleic Acid Line at Different Field Locations in Idaho

5	Location	Percent Fatty Acids				
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>
	Burley	3.3	2.1	77.5	8.1	6.0
	Tetonia	3.5	3.4	77.8	6.5	4.7
	Lamont	3.4	1.9	77.8	7.4	6.5
	Shelley	3.3	2.6	80.0	5.7	4.5

10 Sats=Total Saturate Content

The genetic relationship of the high oleic acid mutation A129 to other oleic desaturases was demonstrated in crosses made to commercial canola cultivars and a low linolenic acid mutation. A129 was crossed to the commercial 15 cultivar Global (C<sub>16:0</sub> - 4.5%, C<sub>18:0</sub> - 1.5%, C<sub>18:1</sub> - 62.9%, C<sub>18:2</sub> - 20.0%, C<sub>18:3</sub> - 7.3%). Approximately 200 F<sub>2</sub> individuals were analyzed for fatty acid composition. The results are summarized in Table 11. The segregation fit 1:2:1 ratio suggesting a single co-dominant gene controlled the 20 inheritance of the high oleic acid phenotype.

TABLE 11  
Genetic Studies of A129 X Global

25	Genotype	C <sub>18:0</sub> Content (%)	Frequency	
			Observed	Expected
	od-od-	77.3	43	47
	od-od+	71.7	106	94
	od+od+	66.1	49	47

A cross between A129 and IMC 01, a low linolenic 30 acid variety (C<sub>16:0</sub> - 4.1%, C<sub>18:0</sub> - 1.9%, C<sub>18:1</sub> - 66.4%, C<sub>18:2</sub> - 18.1%, C<sub>18:3</sub> - 5.7%), was made to determine the inheritance of the oleic acid desaturase and linoleic acid desaturase.

In the  $F_1$  hybrids both the oleic acid and linoleic acid desaturase genes approached the mid-parent values indicating a co-dominant gene actions. Fatty acid analysis of the  $F_2$  individuals confirmed a 1:2:1:2:4:2:1:2:1 segregation of two 5 independent, co-dominant genes (Table 12). A line was selected from the cross of A129 and IMC01 and designated as IMC130 (ATCC deposit no. 75446) as described in U.S. Patent Application No. 08/425,108, incorporated herein by reference.

10

**TABLE 12**  
Genetic Studies of A129 X IMC 01

Genotype	Ratio	Frequency	
		Observed	Expected
od-od-1d-1d-	1	11	12
od-od-1d-1d+	2	30	24
od-od-1d+1d+	1	10	12
od-od+1d-1d-	2	25	24
od-od+1d-1d+	4	54	47
od-od+1d+1d+	2	18	24
15 od+od-1d-1d-	1	7	12
od+od+1d-1d+	2	25	24
20 od+od+1d+1d+	1	8	12

An additional high oleic acid line, designated A128.3, was also produced by the disclosed method. A 50-25 seed bulk analysis of this line showed the following fatty acid composition:  $C_{16:0}$  - 3.5%,  $C_{18:0}$  - 1.8%,  $C_{18:1}$  - 77.3%,  $C_{18:2}$  - 9.0%,  $C_{18:3}$  - 5.6%, FDA Sats - 5.3%, Total Sats - 6.4%. This line also stably maintained its mutant fatty acid composition to the  $M_1$  generation. In multiple locations 30 replicated yield trials, A128 was not significantly different in yield from the parent cultivar Westar.

A129 was crossed to A128.3 for allelism studies. Fatty acid composition of the  $F_2$  seed showed the two lines to be allelic. The mutational events in A129 and A128.3 35 although different in origin were in the same gene.

An additional high oleic acid line, designated M3028.-10 (ATCC 75026), was also produced by the disclosed method in Example 1. A 10-seed bulk analysis of this line showed the following fatty acid composition: C<sub>16:0</sub> - 3.5%, 5 C<sub>18:0</sub> - 1.8%, C<sub>18:1</sub> - 77.3%, C<sub>18:2</sub> - 9.0%, C<sub>18:3</sub> - 5.6%, FDA Saturates - 5.3%, Total Saturates - 6.4%. In a single location replicated yield trial M3028.10 was not significantly different in yield from the parent cultivar Westar.

10

### EXAMPLE 3

#### Low Linoleic Acid Canola

In the studies of Example 1, at the M<sub>3</sub> generation, 80 lines exceeded the lower statistical threshold for linoleic acid ( $\leq$  13.2%). Line W12638.8 had 9.4% linoleic 15 acid. At the M<sub>4</sub> and M<sub>5</sub> generations, its selfed progenies [W12638.8, since designated A133.1 (ATCC 40812)] continued to exceed the statistical threshold for low C<sub>18:2</sub> with linoleic acid levels of 10.2% and 8.4%, respectively. The fatty acid composition of this low linoleic acid mutant, 20 which was stable to the M<sub>5</sub> generation under both field and greenhouse conditions, is summarized in Table 13. In multiple location replicated yield trials, A133 was not significantly different in yield from the parent cultivar Westar. An additional low linoleic acid line, designated 25 M3062.8 (ATCC 75025), was also produced by the disclosed method. A 10-seed bulk analysis of this line showed the following fatty acid composition: C<sub>16:0</sub> - 3.8%, C<sub>18:0</sub> - 2.3%, C<sub>18:1</sub> - 77.1%, C<sub>18:2</sub> - 8.9%, C<sub>18:3</sub> - 4.3%, FDA Sats-6.1%. This line has also stably maintained its mutant fatty acid 30 composition in the field and greenhouse.

TABLE 13

Fatty Acid Composition of a Low  
Linoleic Acid Canola Line Produced by Seed Mutagenesis

5	Genotype	Percent Fatty Acids					
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	Sats <sup>b</sup>
10	Westar	3.9	1.9	67.5	17.6	7.4	7.0
	W12638.8 (M <sub>3</sub> )	3.9	2.3	75.0	9.4	6.1	7.5
15	W12638.8.1 (M <sub>4</sub> )	4.1	1.7	74.6	10.2	5.9	7.1
	A133.1.8 (M <sub>5</sub> )	3.8	2.0	77.7	8.4	5.0	7.0

<sup>a</sup>Letter and numbers up to second decimal point indicate the plant line. Number after second decimal point indicates an individual plant.

<sup>b</sup>Sats=Total Saturate Content

EXAMPLE 4

Low Linolenic and Linoleic Acid Canola

In the studies of Example 1, at the M<sub>3</sub> generation, 20 57 lines exceeded the lower statistical threshold for linolenic acid ( $\leq 5.3\%$ ). Line W14749.8 had 5.3% linolenic acid and 15.0% linoleic acid. At the M<sub>4</sub> and M<sub>5</sub> generations, its selfed progenies [W14749.8, since designated M3032 (ATCC 75021)] continued to exceed the statistical threshold for 25 low C<sub>18:3</sub> with linolenic acid levels of 2.7% and 2.3%, respectively, and for a low sum of linolenic and linoleic acids with totals of 11.8% and 12.5% respectively. The fatty acid composition of this low linolenic acid plus linoleic acid mutant, which was stable to the M<sub>5</sub> generation 30 under both field and greenhouse conditions, is summarized in Table 14. In a single location replicated yield trial M3032

was not significantly different in yield from the parent cultivar (Westar).

TABLE 14

5 Fatty Acid Composition of a Low  
Linolenic Acid Canola Line Produced by Seed Mutagenesis

Genotype	Percent Fatty Acids					
	C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	Sats
Westar	3.9	1.9	67.5	17.6	7.4	7.0
W14749.8 (M <sub>3</sub> )	4.0	2.5	69.4	15.0	5.3	6.5
M3032.8 (M <sub>4</sub> )	3.9	2.4	77.9	9.1	2.7	6.4
M3032.1 (M <sub>5</sub> )	3.5	2.8	80.0	10.2	2.3	6.5

15 Sats=Total Saturate Content

EXAMPLE 5

Canola Lines Q508 and Q4275

Seeds of the *B. napus* line IMC-129 were mutagenized with methyl N-nitrosoguanidine (MNNG). The MNNG treatment 20 consisted of three parts: pre-soak, mutagen application, and wash. A 0.05M Sorenson's phosphate buffer was used to maintain pre-soak and mutagen treatment pH at 6.1. Two hundred seeds were treated at one time on filter paper (Whatman #3M) in a petri dish (100mm x 15mm). The seeds 25 were pre-soaked in 15 mls of 0.05M Sorenson's buffer, pH 6.1, under continued agitation for two hours. At the end of the pre-soak period, the buffer was removed from the plate.

A 10mM concentration of MNNG in 0.05M Sorenson's buffer, pH 6.1, was prepared prior to use. Fifteen ml of 30 10m MNNG was added to the seeds in each plate. The seeds were incubated at 22°C±3°C in the dark under constant

agitation for four (4) hours. At the end of the incubation period, the mutagen solution was removed.

The seeds were washed with three changes of distilled water at 10 minute intervals. The fourth wash was 5 for thirty minutes. This treatment regime produced an LD60 population.

Treated seeds were planted in standard greenhouse potting soil and placed into an environmentally controlled greenhouse. The plants were grown under sixteen hours of 10 light. At flowering, the racemes were bagged to produce selfed seed. At maturity, the M2 seed was harvested. Each M2 line was given an identifying number. The entire MNNG-treated seed population was designated as the Q series.

Harvested M2 seeds was planted in the greenhouse. 15 The growth conditions were maintained as previously described. The racemes were bagged at flowering for selfing. At maturity, the selfed M3 seed was harvested and analyzed for fatty acid composition. For each M3 seed line, approximately 10-15 seeds were analyzed in bulk as described 20 in Example 1.

High oleic-low linoleic M3 lines were selected from the M3 population using a cutoff of >82% oleic acid and <5.0% linoleic. From the first 1600 M3 lines screened for fatty acid composition, Q508 was identified. The Q508 M3 25 generation was advanced to the M4 generation in the greenhouse. Table 15 shows the fatty acid composition of Q508 and IMC 129. The M4 selfed seed maintained the selected high oleic-low linoleic acid phenotype (Table 16).

TABLE 15

Fatty Acid Composition of A129 and High  
Oleic Acid M3 Mutant Q508

	Line #	16:0	18:0	18:1	18:2	18:3
5	A129*	4.0	2.4	77.7	7.8	4.2
	Q508	3.9	2.1	84.9	2.4	2.9

\*Fatty acid composition of A129 is the average of 50 self-pollinated plants grown with the M3 population

10 M<sub>4</sub> generation Q508 plants had poor agronomic qualities in the field compared to Westar. Typical plants were slow growing relative to Westar, lacked early vegetative vigor, were short in stature, tended to be chlorotic and had short pods. The yield of Q508 was very low compared to Westar.

15 The M<sub>4</sub> generation Q508 plants in the greenhouse tended to be reduced in vigor compared to Westar. However, Q508 yields in the greenhouse were greater than Q508 yields in the field.

TABLE 16

20 Fatty Acid Composition of Seed Oil  
from Greenhouse-Grown Q508, IMC 129 and Westar.

	Line	16:0	18:0	18:1	18:2	18:3	FDA Sats
25	IMC 129 <sup>a</sup>	4.0	2.4	77.7	7.8	4.2	6.4
	Westar <sup>b</sup>	3.9	1.9	67.5	17.6	7.4	>5.8
	Q508 <sup>c</sup>	3.9	2.1	84.9	2.4	2.9	6.0

<sup>a</sup>Average of 50 self-pollinated plants

<sup>b</sup>Data from Example 1

<sup>c</sup>Average of 50 self-pollinated plants

Nine other M4 high-oleic low-linoleic lines were also identified: Q3603, Q3733, Q4249, Q6284, Q6601, Q6761, Q7415, Q4275, and Q6676. Some of these lines had good agronomic characteristics and an elevated oleic acid level 5 in seeds of about 80% to about 84%.

Q4275 was crossed to the variety Cyclone. After selfing for seven generations, mature seed was harvested from 93GS34-179, a progeny line of the Q4275 Cyclone cross. Referring to Table 17, fatty acid composition of a bulk seed 10 sample shows that 93GS34 retained the seed fatty acid composition of Q4275. 93GS34-179 also maintained agronomically desirable characteristics.

After more than seven generations of selfing of Q4275, plants of Q4275, IMC 129 and 93GS34 were field grown 15 during the summer season. The selections were tested in 4 replicated plots (5 feet X 20 feet) in a randomized block design. Plants were open pollinated. No selfed seed was produced. Each plot was harvested at maturity, and a sample of the bulk harvested seed from each line was analyzed for 20 fatty acid composition as described above. The fatty acid compositions of the selected lines are shown in Table 17.

TABLE 17  
Fatty Acid Composition of  
Field Grown IMC 129, Q4275 and 93GS34 Seeds

Line	Fatty Acid Composition (%)					
	C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	FDA Sats
IMC 129	3.3	2.4	76.7	8.7	5.2	5.7
Q4275	3.7	3.1	82.1	4.0	3.5	6.8
93GS34-179	2.6	2.7	85.0	2.8	3.3	5.3

The results shown in Table 17 show that Q4275 maintained the selected high oleic - low linoleic acid phenotype under field conditions. The agronomic characteristics of Q4275 plants were superior to those of 5 Q508.

M<sub>4</sub> generation Q508 plants were crossed to a dihaploid selection of Westar, with Westar serving as the female parent. The resulting F<sub>1</sub> seed was termed the 92EF population. About 126 F<sub>1</sub> individuals that appeared to have 10 better agronomic characteristics than the Q508 parent were selected for selfing. A portion of the F<sub>2</sub> seed from such individuals was replanted in the field. Each F<sub>2</sub> plant was selfed and a portion of the resulting F<sub>3</sub> seed was analyzed for fatty acid composition. The content of oleic acid in F<sub>3</sub> 15 seed ranged from 59 to 79%. No high oleic (>80%) individuals were recovered with good agronomic type.

A portion of the F<sub>2</sub> seed of the 92EF population was planted in the greenhouse to analyze the genetics of the Q508 line. F<sub>3</sub> seed was analyzed from 380 F<sub>2</sub> individuals. 20 The C<sub>18:1</sub> levels of F<sub>3</sub> seed from the greenhouse experiment is depicted in Figure 1. The data were tested against the hypothesis that Q508 contains two mutant genes that are semi-dominant and additive: the original IMC 129 mutation as well as one additional mutation. The hypothesis also 25 assumes that homozygous Q508 has greater than 85% oleic acid and homozygous Westar has 62-67% oleic acid. The possible genotypes at each gene in a cross of Q508 by Westar may be designated as:

AA = Westar Fad2<sup>a</sup>  
30 BB = Westar Fad2<sup>b</sup>  
aa = Q508 Fad2<sup>a-</sup>  
bb = Q508 Fad2<sup>b-</sup>

Assuming independent segregation, a 1:4:6:4:1 ratio of phenotypes is expected. The phenotypes of heterozygous plants are assumed to be indistinguishable and, thus, the data were tested for fit to a 1:14:1 ratio of homozygous 5 Westar: heterozygous plants: homozygous Q508.

Phenotypic Ratio	# of Westar Alleles	Genotype
10	1	AABB (Westar)
	4	AABb, AaBB, AABb, AaBB
	6	AaBb, AAbb, AaBb, AaBb, aaBB, AaBb
	4	Aabb, aaBb, Aabb, aaBb
	1	aabb (Q508)

Using Chi-square analysis, the oleic acid data fit a 1:14:1 ratio. It was concluded that Q508 differs from 15 Westar by two major genes that are semi-dominant and additive and that segregate independently. By comparison, the genotype of IMC 129 is aaBB.

The fatty acid composition of representative F3 individuals having greater than 85% oleic acid in seed oil 20 is shown in Table 18. The levels of saturated fatty acids are seen to be decreased in such plants, compared to Westar.

**TABLE 18**  
92EF F<sub>3</sub> Individuals with >85% C<sub>18:1</sub> in Seed Oil

F3 Plant Identifier	Fatty Acid Composition (%)						
	C16:0	C18:0	C18:1	C18:2	C18:3	FDASA	
5	+38068	3.401	1.582	85.452	2.134	3.615	4.983
	+38156	3.388	1.379	85.434	2.143	3.701	4.767
	+38171	3.588	1.511	85.289	2.367	3.425	5.099
	+38181	3.75	1.16	85.312	2.968	3.819	4.977
	+38182	3.529	0.985	85.905	2.614	3.926	4.56
10	+38191	3.364	1.039	85.737	2.869	4.039	4.459
	+38196	3.557	1.182	85.054	2.962	4.252	4.739
	+38202	3.554	1.105	86.091	2.651	3.721	4.713
	+38220	3.093	1.16	86.421	1.931	3.514	4.314
	+38236	3.308	1.349	85.425	2.37	3.605	4.718
15	+38408	3.617	1.607	85.34	2.33	3.562	5.224
	+38427	3.494	1.454	85.924	2.206	3.289	4.948
	+38533	3.64	1.319	85.962	2.715	3.516	4.959

**EXAMPLE 6**

20 Leaf and Root Fatty Acid Profiles of Canola  
Lines IMC-129, Q508, and Westar

Plants of Q508, IMC 129 and Westar were grown in the greenhouse. Mature leaves, primary expanding leaves, petioles and roots were harvested at the 6-8 leaf stage,

frozen in liquid nitrogen and stored at -70°C. Lipid extracts were analyzed by GLC as described in Example 1. The fatty acid profile data are shown in Table 19.

The data in Table 19 indicate that total leaf lipids 5 in Q508 are higher in  $C_{18:1}$  content than the  $C_{18:2}$  plus  $C_{18:3}$  content. The reverse is true for Westar and IMC 129. The difference in total leaf lipids between Q508 and IMC 129 is consistent with the hypothesis that a second Fad2 gene is mutated in Q508.

10 The  $C_{16:3}$  content in the total lipid fraction was about the same for all three lines, suggesting that the plastid FadC gene product was not affected by the Q508 mutations. To confirm that the FadC gene was not mutated, chloroplast lipids were separated and analyzed. No changes 15 in chloroplast  $C_{16:1}$ ,  $C_{16:2}$  or  $C_{16:3}$  fatty acids were detected in the three lines. The similarity in plastid leaf lipids among Q508, Westar and IMC 129 is consistent with the hypothesis that the second mutation in Q508 affects a microsomal Fad2 gene and not a plastid FadC gene.

TABLE 19

	MATURE LEAF			EXPANDING LEAF			PETIOLE			ROOT		
	West.	129	3Q508	West.	129	3Q508	West.	129	3Q508	West.	129	3Q508
5	16:0	12.1	11.9	10.1	16.4	16.1	11.3	21.7	23.5	11.9	21.1	21.9
	16:1	0.8	0.6	1.1	0.7	0.6	1.1	1.0	1.3	1.4	-	-
	16:2	2.3	2.2	2.0	2.8	3.1	2.8	1.8	2.2	1.8	-	-
	16:3	14.7	15.0	14.0	6.3	5.4	6.9	5.7	4.6	5.7	-	-
	18:0	2.2	1.6	1.2	2.5	2.8	1.5	3.7	4.0	1.6	3.6	2.9
	18:1	2.8	4.9	16.7	3.8	8.3	38.0	4.9	12.9	46.9	3.5	6.1
	18:2	12.6	11.5	6.8	13.3	13.8	4.9	20.7	18.3	5.2	28.0	30.4
	18:3	50.6	50.3	46.0	54.2	50.0	33.5	40.4	33.2	25.3	43.8	38.7
												12.3

10

EXAMPLE 7

Sequences of Mutant and Wild-Type Delta-12 Fatty Acid Desaturases from *B. napus*

Primers specific for the FAD2 structural gene were used to clone the entire open reading frame (ORF) of the D and F delta-12 desaturase genes by reverse transcriptase polymerase chain reaction (RT-PCR). RNA from seeds of IMC 129, Q508 and Westar plants was isolated by standard methods and was used as template. The RT-amplified fragments were used for nucleotide sequence determination. The DNA sequence of each gene from each line was determined from both strands by standard dideoxy sequencing methods.

Sequence analysis revealed a G to A transversion at nucleotide 316 (from the translation initiation codon) of the D gene in both IMC 129 and Q508, compared to the sequence of Westar. The transversion changes the codon at this position from GAG to AAG and results in a non-conservative substitution of glutamic acid, an acidic residue, for lysine a basic residue. The presence of the

same mutation in both lines was expected since the Q508 line was derived from IMC 129. The same base change was also detected in Q508 and IMC 129 when RNA from leaf tissue was used as template.

5 The G to A mutation at nucleotide 316 was confirmed by sequencing several independent clones containing fragments amplified directly from genomic DNA of IMC 129 and Westar. These results eliminated the possibility of a rare mutation introduced during reverse transcription and PCR in 10 the RT-PCR protocol. It was concluded that the IMC 129 mutant is due to a single base transversion at nucleotide 316 in the coding region of the D gene of rapeseed microsomal delta 12-desaturase.

15 A single base transition from T to A at nucleotide 515 of the F gene was detected in Q508 compared to the Westar sequence. The mutation changes the codon at this position from CTC to CAC, resulting in the non-conservative substitution of a non-polar residue, leucine, for a polar residue, histidine, in the resulting gene product. No 20 mutations were found in the F gene sequence of IMC 129 compared to the F gene sequence of Westar.

25 These data support the conclusion that a mutation in a delta-12 desaturase gene sequence results in alterations in the fatty acid profile of plants containing such a mutated gene. Moreover, the data show that when a plant line or species contains two delta-12 desaturase loci, the fatty acid profile of an individual having two mutated loci differs from the fatty acid profile of an individual having one mutated locus.

30 The mutation in the D gene of IMC 129 and Q508 mapped to a region having a conserved amino acid motif (His-Xaa-Xaa-Xaa-His) found in cloned delta-12 and delta-15 membrane bound-desaturases (Table 20).

TABLE 20

Alignment of Amino Acid Sequences  
of Cloned Canola Membrane Bound-Desaturases

Desaturase Gene	Sequence <sup>a</sup>	Position
Canola-fad2-D (mutant)	AHKCGH	109-114
	AHECGH	109-114
	AHECGH	109-114
	<u>GHDCAH</u>	170-175
Canola-fad3 (mutant)		
	<u>GHKCGH</u>	94-99
	<u>GHDCGH</u>	94-99
Canola-FadD	<u>GHDCGH</u>	125-130

(FadD = Plastid delta 15, Fad3 = Microsomal delta-15),  
(FadC = Plastid delta-12, Fad2 = Microsomal delta-12)

<sup>a</sup> One letter amino acid code; conservative substitutions are underlined; non-conservative substitutions are in bold.

EXAMPLE 8

Transcription and Translation of Microsomal Delta-12  
Fatty Acid Desaturases

Transcription *in vivo* was analyzed by RT-PCR  
20 analysis of stage II and stage III developing seeds and leaf tissue. The primers used to specifically amplify delta-12 desaturase F gene RNA from the indicated tissues were sense primer 5'-GGATATGATGGTGAAAGA-3' and antisense primer 5'-TCTTCACCACATCATCATATCC-3'. The primers used to specifically amplify delta-12 desaturase D gene RNA from the indicated tissues were sense primer 5'-GTTATGAAGCAAAGAAGAAC-3' and antisense primer 5'-GTTTCTTCTTGCTTCATAAC-3'. The results indicated that mRNA of both the D and F gene was expressed

in seed and leaf tissues of IMC 129, Q508 and wild type Westar plants.

5 *In vitro* transcription and translation analysis showed that a peptide of about 46 kD was made. This is the expected size of both the D gene product and the F gene product, based on sum of the deduced amino acid sequence of each gene and the cotranslational addition of a microsomal membrane peptide.

10 These results rule out the possibility that non-sense or frameshift mutations, resulting in a truncated polypeptide gene product, are present in either the mutant D gene or the mutant F gene. The data, in conjunction with the data of Example 7, support the conclusion that the mutations in Q508 and IMC 129 are in delta-12 fatty acid 15 desaturase structural genes encoding desaturase enzymes, rather than in regulatory genes.

#### EXAMPLE 9

##### Development of Gene-Specific PCR Markers

Based on the single base change in the mutant D gene 20 of IMC 129 described in above, two 5' PCR primers were designed. The nucleotide sequence of the primers differed only in the base (G for Westar and A for IMC 129) at the 3' end. The primers allow one to distinguish between mutant fad2-D and wild-type Fad2-D alleles in a DNA-based PCR 25 assay. Since there is only a single base difference in the 5' PCR primers, the PCR assay is very sensitive to the PCR conditions such as annealing temperature, cycle number, amount, and purity of DNA templates used. Assay conditions have been established that distinguish between the mutant 30 gene and the wild type gene using genomic DNA from IMC 129 and wild type plants as templates. Conditions may be further optimized by varying PCR parameters, particularly

with variable crude DNA samples. A PCR assay distinguishing the single base mutation in IMC 129 from the wild type gene along with fatty acid composition analysis provides a means to simplify segregation and selection analysis of genetic 5 crosses involving plants having a delta-12 fatty acid desaturase mutation.

EXAMPLE 10

Transformation with Mutant and Wild Type Fad3 Genes

10 *B. napus* cultivar Westar was transformed with mutant and wild type Fad3 genes to demonstrate that the mutant Fad3 gene for canola cytoplasmic linoleic desaturase delta-15 desaturase is nonfunctional. Transformation and regeneration were performed using disarmed *Agrobacterium* 15 *tumefaciens* essentially following the procedure described in WO 94/11516.

Two disarmed *Agrobacterium* strains were engineered, each containing a Ti plasmid having the appropriate gene linked to a seed-specific promoter and a corresponding termination sequence. The first plasmid, pIMC110, was 20 prepared by inserting into a disarmed Ti vector the full length wild type Fad3 gene in sense orientation (nucleotides 208 to 1336 of SEQ ID 6 in WO 93/11245), flanked by a napin promoter sequence positioned 5' to the Fad3 gene and a napin 25 termination sequence positioned 3' to the Fad3 gene. The rapeseed napin promoter is described in EP 0255378.

The second plasmid, pIMC205, was prepared by inserting a mutated Fad3 gene in sense orientation into a disarmed Ti vector. The mutant sequence contained mutations at nucleotides 411 and 413 of the microsomal Fad3 gene 30 described in WO93/11245, thus changing the sequence for codon 96 from GAC to AAG. The amino acid at codon 96 of the gene product was thereby changed from aspartic acid to lysine. See Table 20. A bean (*Phaseolus vulgaris*)

phaseolin (7S seed storage protein) promoter fragment of 495 base pairs, starting with 5'-TGGTCTTTGGT-3', was placed 5' to the mutant Fad3 gene and a phaseolin termination sequence was placed 3' to the mutant Fad3 gene. The phaseolin 5 sequence is described in Doyle et al., (1986) J. Biol. Chem. 261:9228-9238) and Slightom et al., (1983) Proc. Natl. Acad. Sci. USA 80:1897-1901.

The appropriate plasmids were engineered and transferred separately to *Agrobacterium* strain LBA4404. 10 Each engineered strain was used to infect 5 mm segments of hypocotyl explants from Westar seeds by cocultivation. Infected hypocotyls were transferred to callus medium and, subsequently, to regeneration medium. Once discernable stems formed from the callus, shoots were excised and 15 transferred to elongation medium. The elongated shoots were cut, dipped in Rootone™, rooted on an agar medium and transplanted to potting soil to obtain fertile T1 plants. T2 seeds were obtained by selfing the resulting T1 plants.

Fatty acid analysis of T2 seeds was carried out as 20 described above. The results are summarized in Table 21. Of the 40 transformants obtained using the pIMC110 plasmid, 17 plants demonstrated wild type fatty acid profiles and 16 demonstrated overexpression. A proportion of the 25 transformants are expected to display an overexpression phenotype when a functioning gene is transformed in sense orientation into plants.

Of the 307 transformed plants having the pIMC205 gene, none exhibited a fatty acid composition indicative of overexpression. This result indicates that the mutant fad3 30 gene product is non-functional, since some of the transformants would have exhibited an overexpression phenotype if the gene product were functional.

**TABLE 21**  
**Overexpression and Co-suppression Events in**  
**Westar Populations Transformed with pIMC205 or pIMC110.**

5

Construct	Number of Transformants	$\alpha$ -Linolenic Acid Range(%)	Overexpression Events (>10% linolenic)	Co-Suppression Events (<4.0% linolenic)	Wild Type Events
pIMC110	40	2.4 - 20.6	16	7	17
pIMC205	307	4.6 - 10.4	0	0	307

10 Fatty acid compositions of representative transformed plants are presented in Table 22. Lines 652-09 and 663-40 are representative of plants containing pIMC110 and exhibiting an overexpression and a co-suppression phenotype, respectively. Line 205-284 is representative of plants containing pIMC205 and having the mutant fad3 gene.

15

**TABLE 22**  
**Fatty Acid Composition of T2 Seed**  
**From Westar Transformed With pIMC205 or pIMC110.**

20

Line	Fatty Acid Composition (%)				
	C16:0	C18:0	C18:1	C18:2	C18:3
652-09 pIMC110 overexpression	4.7	3.3	65.6	8.1	14.8
663-40 pIMC110 co-suppression	4.9	2.1	62.5	23.2	3.6
205-284 pIMC205	3.7	1.8	68.8	15.9	6.7

25

**EXAMPLE 11**

Sequences of Wild Type and Mutant Fad2-D and Fad2-F

High molecular weight genomic DNA was isolated from leaves of Q4275 plants (Example 5). This DNA was used as template for amplification of Fad2-D and Fad2-F genes by polymerase chain reaction (PCR). PCR amplifications were carried out in a total volume of 100  $\mu$ l and contained 0.3  $\mu$ g genomic DNA, 200  $\mu$ M deoxyribonucleoside triphosphates, 3 mM

MgSO<sub>4</sub>, 1-2 Units DNA polymerase and 1X Buffer (supplied by the DNA polymerase manufacturer). Cycle conditions were: 1 cycle for 1 min at 95°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 73°C.

5 The Fad2-D gene was amplified once using Elongase® (Gibco-BRL). PCR primers were:

CAUCAUCAUCAUCAUTCTTCGTAGGGTTCATCG and CUACUACUACUATCATAGAAGAGAAAGGTTCA for the 5' and 3' ends of the gene, respectively.

10 The Fad2-F gene was independently amplified 4 times, twice with Elongase® and twice with Taq polymerase (Boehringer Mannheim). The PCR primers used were: 5' CAUCAUCAUCAUCATGGGTGCACGTGGAAGAA3' and 5' CUACUACUACUATCTTCACCATCATCATATCC3' for the 5' and 3' ends 15 of the gene, respectively.

Amplified DNA products were resolved on an agarose gel, purified by JetSorb® and then annealed into pAMP1 (Gibco-BRL) via the (CAU)<sub>4</sub> and (CUA)<sub>4</sub> sequences at the ends of the primers, and transformed into *E. coli* DH5 $\alpha$ .

20 The Fad2-D and Fad2-F inserts were sequenced on both strands with an ABI PRISM 310 automated sequencer (Perkin-Elmer) following the manufacturer's directions, using synthetic primers, AmpliTaq® DNA polymerase and dye terminator.

25 The Fad2-D gene was found to have an intron upstream of the ATG start codon. As expected, the coding sequence of the gene contained a G to A mutation at nucleotide 316, derived from IMC 129 (Fig. 2).

30 A single base transversion from G to A at nucleotide 908 was detected in the F gene sequence of the Q4275 amplified products, compared to the wild type F gene sequence (Fig. 2). This mutation changes the codon at amino acid 303 from GGA to GAA, resulting in the non-conservative

substitution of a glutamic acid residue for a glycine residue (Table 3 and Fig. 3). Expression of the mutant Q4275 Fad2-F delta-12 desaturase gene in plants alters the fatty acid composition, as described hereinabove.

5 To the extent not already indicated, it will be understood by those of ordinary skill in the art that any one of the various specific embodiments herein described and illustrated may be further modified to incorporate features shown in other of the specific embodiments.

10 The foregoing detailed description has been provided for a better understanding of the invention only and no unnecessary limitation should be understood therefrom as some modifications will be apparent to those skilled in the art without deviating from the spirit and scope of the  
15 appended claims.